Inhibition of the Anticoagulant Activity of Protein S by Prothrombin

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Abstract

Protein S is a vitamin K-dependent protein cofactor to the anticoagulant, activated protein C (APC). This study examines the inhibition of human protein S anticoagulant activity by prothrombin.

In the absence of protein S, the anticoagulant activity of APC measured in a Factor X_a recalcification time, was comparable using normal or plasma adsorbed with Al(OH)₃. Protein S was an effective cofactor to APC in Al(OH)₃-adsorbed plasma, but was significantly less active in normal plasma. Analysis of the difference in the two plasmas revealed that normal plasma contained an inhibitor to the anticoagulant activity of protein S that was removed by Al(OH)₃ adsorption. Purification of this inhibitory activity demonstrated that it was mediated by the vitamin K-dependent protein, prothrombin.

Prothrombin purified by conventional techniques caused immediate, dose-dependent inhibition of the cofactor activity of protein S in the presence of phospholipids or platelets, but had no effect on the anticoagulant activity of APC. The inhibition was demonstrable using a Factor X_a recalcification time, and studies of the rates of inactivation of purified Factor V_a. Increasing concentrations of protein S overcame the inhibition by prothrombin and kinetic analysis of the interaction demonstrated that prothrombin acted as a competitive inhibitor to protein S. Immunoabsorption of prothrombin from plasma using immobilized antiprothrombin antibodies was associated with the complete removal of the protein S inhibitory activity.

We conclude that the anticoagulant activity of protein S is modulated by prothrombin and that this may represent another regulatory mechanism of the natural anticoagulant system.

Introduction

Protein S is a vitamin K-dependent glycoprotein cofactor to the serine protease-activated protein C (APC)¹ (1). APC inactivates Factors V_a and VIII_a in a Ca²⁺- and lipid-dependent reaction (2, 3). Studies using the bovine system have demonstrated that protein S forms a 1:1 molar complex with APC, resulting in the increased affinity of the protease for the platelet or phospholipid surface (4, 5). The importance of these proteins in the regulation of hemostasis in man is highlighted

by reports of recurrent venous thrombosis in individuals who are deficient in either proteins (6, 7).

Unlike other coagulation proteins, protein S circulates in plasma in an active form and does not require activation before partaking in the coagulation reaction. A number of regulatory mechanisms for the control of protein S anticoagulant activity have been proposed. Thrombin causes the loss of protein S activity, concomitant with the cleavage of a peptide near the amino-terminal end of the molecule (8–10). The *in vivo* significance of this reaction in man is unclear in view of the observation that physiological concentrations of Ca²⁺ and the endothelial protein thrombomodulin, inhibit the effects of thrombin (11). More recently, Dahlback has shown that the cofactor activity of protein S was inhibited by the complement regulatory protein, C4b-binding protein (12). In other studies, protein S has been shown to bind to platelets and subsequently cleaved and inactivated by a Ca²⁺-dependent protease (13). This cellular control of protein S activity may represent a physiological regulatory event.

In this study, we identify a rapidly acting plasma inhibitor of the cofactor activity of protein S. Our results suggest that the inhibitor is prothrombin, which in physiological concentrations, acts as a competitive inhibitor to the cofactor activity of protein S.

Methods

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) except for the following: NaCl, EDTA, and glycine, (from Ajax Chemical Company, Australia), SDS (Pierce Chemical Corp., Rockford, IL), acrylamide (Bio-Rad Laboratories, Richmond, CA), and platelin (General Diagnostics, Morris Plains, NJ).

Coagulation factors were of human origin. Protein C (14), protein S (8), Factor V (15), prothrombin, Factor X (16, 17), thrombin (17), and fibrinogen (18) were purified and or activated as previously described.

SDS-PAGE was performed according to Laemmli (19), using a 5% stacking gel and a 10% running gel. Before application to the gel, the samples were reduced by boiling in the presence of 5% mercaptoethanol.

Washed platelets were prepared as previously described (13).

Functional protein S assay. This assay is based on a Factor X_a recalcification time (11), and relies upon the ability of protein S to enhance the anticoagulant activity of APC. To 50 μ l of plasma or Al(OH)₃-adsorbed plasma was added 100 μ l of 1.2 μ M prothrombin containing 10% platelin (reconstituted according to the manufacturers' instructions), and 50 μ l of buffer or 25 μ l of APC or protein S. The buffer used was 20 mM Tris (pH 7.4) containing 0.15 M NaCl and 0.1% gelatine. Clotting was initiated by the addition of 50 μ l of 8 nM Factor X_a and 25 μ l of 80 mM Ca²⁺. The time was recorded in triplicate using a four-channel coagulator (Behnk Elektronik, Hamburg, Federal Republic of Germany).

Factor V coagulant activity was measured in a one-stage Factor V assay as previously described (20). The assay mixture consisted of 50 μ l each of (a) 3 mg/ml human fibrinogen, (b) 100 μ g/ml human prothrombin and 100 μ M phospholipid vesicles (prepared as described in reference 20), (c) 25 ng/ml human Factor X_a, and (d) sample. The assay buffer used to dilute the assay reagents consisted of 20 mM Tris

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1. Abbreviations used in this paper: APC, serine protease-activated protein C.

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(pH 7.4) containing 0.1 M NaCl and 0.1% gelatine. Clotting was initiated by the addition of 50 μ l of 40 mM CaCl_2 . The time was recorded in duplicate using a fibrometer (Becton-Dickinson & Co., Oxnard, CA). Standard curves were prepared using pooled plasma collected from healthy volunteers.

Polyclonal prothrombin antibodies were obtained from rabbits immunized with 50 μ g prothrombin injected at multiple intradermal sites. The antiprothrombin antibody was isolated from the rabbit antiserum using a Protein A Sepharose column. The IgG was then dialyzed against 0.1 M Hepes pH 7.4.

Prothrombin-deficient plasma was prepared by coupling anti-prothrombin IgG to Affigel 10 (Bio-Rad Laboratories) for 10 h at 4°C. After the coupling reaction, residual active esters were blocked with 0.1 M glycine (pH 8.0). The gel was washed with 20 mM Tris pH 7.4, containing 0.15 M NaCl, and used to prepare prothrombin-depleted plasma. For this purpose the gel (5 ml) was incubated with 5 ml of citrated plasma at room temperature for 4 h. At the end of this time, the plasma was collected, tested for its prothrombin content, and used as a source of prothrombin-depleted plasma.

Laurell rocket immunoassays of prothrombin in normal and prothrombin-depleted plasma were performed as previously described (21).

Purification of the inhibitor to protein S activity. Citrated plasma (200 ml) was thawed at 37°C and all subsequent purification steps performed at 4°C. 1 M BaCl_2 (8 ml/100 ml of plasma) was added in a dropwise fashion and stirred for 1 h at 4°C. The precipitate was separated by centrifugation at 10,000 g for 15 min. The supernatant was discarded and the pellet was washed twice in 0.15 M NaCl, resuspended in 0.25 M EDTA (150 ml/liter of plasma), and stirred for 1 h. The protein was dialyzed overnight against 0.02 M imidazole (pH 6.0) and after clarification by centrifugation, applied to a heparin agarose column equilibrated in the same buffer. The protein S inhibitory activity did not bind to the column, and was present in the flowthrough. Fractions containing peak inhibitory activity were pooled and applied to a DEAE-Sepharose column equilibrated with 20 mM Tris (pH 7.4) containing 0.15 M NaCl. The flowthrough fractions were collected and the column was washed until the protein content of the effluent was < 20 μ g/ml. The column was then developed using a linear NaCl gradient (0.15–0.5 M NaCl) in the same buffer and 6-ml fractions were collected. Fractions of interest were pooled, dialyzed against 0.02 M Tris (pH 7.4) containing 0.15 M NaCl, and applied to a blue Sepharose column equilibrated with the same buffer. The inhibitor bound to the column and was eluted using a linear gradient (0.15–0.30 M) NaCl. Eluting fractions were assessed for purity using SDS-PAGE. Throughout the purification, fractions of interest were followed by testing for the presence of inhibitory activity against the cofactor properties of protein S using the functional protein S anticoagulant assay described above. Typically, 25 μ l of appropriately diluted fraction was tested and the concentration of APC and protein S used in the assay was 2 and 75 nM, respectively.

Results

The prothrombin time of $\text{Al}(\text{OH})_3$ -adsorbed plasma was > 300 s, and the protein S and Factor X content was < 0.5 μ g/ml as measured by a Laurell immunoelectrophoretic assay. Prothrombin could not be detected in the adsorbed plasma using an immunoelectrophoretic assay sensitive to 1 μ g/ml prothrombin. Similarly, protein C was undetectable using an RIA with a detection limit of 0.2 μ g/ml. The adsorption process did not, however, affect the Factor X_a recalcification time, which remained similar to the starting plasma (40 $\pm$ 2 s).

The addition of increasing concentrations of APC to normal or adsorbed plasma resulted in a linear and comparable prolongation of the clotting time (Fig. 1). These results were surprising, in view of the significant difference in the level of

Figure 1. Anticoagulant activity of APC in normal and $\text{Al}(\text{OH})_3$ -adsorbed plasma. APC-mediated anticoagulant activity was measured using a Factor X_a recalcification time of ($\blacktriangle$) normal or ($\triangle$) $\text{Al}(\text{OH})_3$ -adsorbed plasma. Details of coagulation assay are presented in Methods.

protein S in the two plasmas, and the known potentiating effects of this protein on the activity of APC.

The effect of exogenously added protein S on the anticoagulant activity of APC in the two forms of plasma is demonstrated in Fig. 2. APC (0.5 nM), caused minimal prolongation of the clotting time (2–5 s). The addition of increasing concentrations of protein S to the adsorbed plasma resulted in significant potentiation of the anticoagulant activity of APC. This effect was less marked when intact plasma was used. Using 0.5 nM APC, a 10-s prolongation of the clotting time of adsorbed plasma was obtained with 10 nM protein S. A comparable prolongation of the clotting time of normal plasma required the addition of 220 nM protein S. Similar results were obtained when BaCl_2 -adsorbed plasma was substituted for $\text{Al}(\text{OH})_3$ -adsorbed plasma. The different cofactor activity of protein S in the two plasmas is even greater, if one considers the contribution of endogenous protein S present in the normal plasma. Collectively, these results suggest that adsorption of plasma by $\text{Al}(\text{OH})_3$ or BaCl_2 removes a molecule that interferes with the expression of the cofactor activity of protein S.

Figure 2. Anticoagulant activity of protein S in normal and $\text{Al}(\text{OH})_3$ -adsorbed plasma. Functional activity of protein S was measured using a Factor X_a recalcification time in the presence of 0.5 nM APC. The prolongation of the clotting time represents the contribution of protein S minus the effect of APC alone. $\blacktriangle$, normal plasma; $\triangle$, $\text{Al}(\text{OH})_3$ -adsorbed plasma.

The possibility of an inhibitor to the anticoagulant activity of protein S, present in plasma and removed by $\text{Al}(\text{OH})_3$ -adsorption, was supported by experiments demonstrated in Fig. 3. In these studies, an increasing percentage of normal plasma was added to $\text{Al}(\text{OH})_3$ -adsorbed plasma and a Factor X_a recalcification time was performed in the presence of 2 nM APC and 85 nM protein S. The addition of increasing amounts of normal plasma to the adsorbed plasma was associated with inhibition of the anticoagulant activity of protein S. Maximum inhibition was observed in the presence of an equal volume of normal and adsorbed plasma. In control studies, normal plasma did not inhibit the anticoagulant activity of APC in the absence of added protein S, and the control clotting time in the absence of APC or protein S remained the same, no matter what percentage normal plasma was used.

We have previously demonstrated that protein S binds to platelets and is subsequently cleaved and inactivated by a Ca^{2+} -dependent membrane protease (13). To eliminate possible contamination of the plasma used in our studies with platelet fragments or membranes, the plasma was centrifuged at 100,000 *g* for 6 h and the supernatant tested for its ability to inhibit the cofactor activity of protein S. The effects of normal plasma on protein S persisted, indicating that it was not mediated via platelet membranes.

Previous studies by Dahlback (12), which we have also confirmed (unpublished observations), indicate that the inhibition of protein S by C4b-binding protein is not an immediate event. The protein S inhibition noted in experiments described in this paper was immediate and was not increased by preincubation of the plasma with protein S. Hence it is unlikely to be mediated via binding of protein S to the C4b-binding protein.

Purification of the inhibitor to protein S was performed as described in Methods. At each chromatographic step, fractions were assayed for protein content and their ability to inhibit the anticoagulant activity of protein S. As expected, the inhibitor was removed by BaCl_2 adsorption of plasma. It did not bind to the heparin agarose column and the activity was recovered in the flowthrough fractions. The inhibitor bound to the anion-exchange column (DEAE Sepharose) and was recovered in the major protein peak eluting with the salt gradient. This peak contained primarily prothrombin as determined by SDS-PAGE. After dialysis, the material eluting off the DEAE-Seph-

arose was applied to a blue Sepharose affinity step, to which the inhibitor completely bound. Elution with NaCl resulted in a major protein peak and corresponding inhibitory activity. SDS-PAGE of the fractions demonstrated a single band with a M_r of 72,000 D. Fractions from this step (at a final concentration of 100 $\mu\text{g}/\text{ml}$), when added to the Factor X_a recalcification time in the absence of APC and protein S, did not shorten the clotting time. Similarly, this material did not alter the anticoagulant activity of APC in the absence of protein S.

To rule out the possibility that a minor contaminant not visualized by the Coomassie brilliant blue stain was responsible for the inhibition of protein S activity, the purified material was applied to a gel filtration column and eluting fractions subjected to SDS-PAGE and assays of inhibitory activity (Fig. 4, A and B). A single protein peak was obtained that corresponded to the elution profile of the inhibitory activity. The chromatographic behavior of the inhibitor and its appearance on SDS-PAGE is identical to prothrombin. The faint lower molecular weight bands visible on reduced SDS-PAGE, repre-

Figure 3. Effect of normal plasma on the cofactor activity of protein S in adsorbed plasma. Factor X_a recalcification time was performed using 50 μl of $\text{Al}(\text{OH})_3$ -adsorbed plasma containing the indicated percentage of normal plasma in the presence of 2 nM APC and 85 nM protein S. The prolongation of the clotting time represents the contribution of protein S minus that seen with APC alone.

Figure 4. (A) Gel filtration of the purified protein S inhibitor on Sephadex G 100. 3 mg of purified material eluting off the blue Sepharose affinity column was applied to a 1.25 $\times$ 20 cm Sephadex G 100 column equilibrated in 20 mM Tris (pH 7.4) containing 0.15 M NaCl. 2-ml fractions were collected. 25 μl of the eluting fraction was assessed for protein S inhibitory activity using the Factor X_a recalcification time of $\text{Al}(\text{OH})_3$ -adsorbed plasma described in Methods. The clotting assay was performed in the presence of 2.0 nM APC, 100 nM protein S, and 50 μl of test fraction. The prolongation of the clotting time represents the contribution of protein S minus the effect of APC alone. (B) SDS-PAGE of fractions eluting from gel filtration column. Sample 25- μl of the indicated fractions corresponding to the gel filtration experiments shown in Fig. 4 A, were boiled in SDS reducing buffer and subjected to reduced SDS-PAGE as described in Methods. SM, starting material before application to the gel filtration column. FN, fraction number.

sent partial activation products of prothrombin, as the gel filtration step was performed in the absence of protease inhibitors. These bands reacted with a polyclonal anti-prothrombin antibody in a Western blotting system, confirming that they represent prothrombin breakdown products.

To confirm that the inhibitory activity was due to prothrombin, the effect of increasing concentrations of conventionally purified prothrombin (purified according to reference 16) on the anticoagulant activity of protein S, was examined (Fig. 5). A marked dose-dependent inhibition of the cofactor activity of protein S was observed. Prothrombin, used at a concentration of $0.6 \mu\text{M}$ (approximately half the plasma concentration), caused a fivefold inhibition of the cofactor potential of protein S. This prothrombin concentration did not alter the baseline control clotting time and did not inhibit the anticoagulant activity of APC in the absence of protein S.

The effect of increasing the concentration of protein S on the inhibitory activity of prothrombin is shown in Fig. 6. Double reciprocal plot analysis of the data, revealed that prothrombin competitively inhibited the anticoagulant activity of protein S. In the presence of $0.4 \mu\text{M}$ prothrombin (the prothrombin concentration used in the Factor X_a recalcification time), the K_m of protein S was 15 nM and this was altered to 240 nM in the presence of $1.6 \mu\text{M}$ prothrombin (normal plasma concentration of prothrombin).

The ability of prothrombin to inhibit the cofactor activity of protein S was also assessed using a purified Factor V_a inactivation assay in the presence of platelets or phospholipids. APC in concentrations as little as 0.3 nM resulted in a rapid inactivation of Factor V_a in the presence of phospholipid. The inactivation curve was biphasic, with 60% of the starting material remaining after 30 s incubation, which dropped to 20% after 4 min incubation. With the addition of $0.3 \mu\text{M}$ protein S, acceleration of the inactivation of Factor V_a was observed, with the Factor V_a level falling to 25% after 30 s and to 5% after 4 min incubation. When prothrombin ($0.5 \mu\text{M}$) was included in the reaction, the cofactor activity of protein S was significantly inhibited, and the Factor V_a inactivation was similar to the curve obtained using APC alone (Fig. 7). Lower concentration of prothrombin ($0.3 \mu\text{M}$ or less), did not affect the cofactor activity of protein S. When platelets $5 \times 10^7/\text{ml}$ were used as a surface for the inactivation of Factor V_a , prothrombin caused a similar inhibition of the cofactor activity of protein S. In control studies prothrombin in concentrations up to

Figure 5. Effect of purified prothrombin on the cofactor activity of protein S. The effect of purified prothrombin on the activity of protein S was assessed using a Factor X_a recalcification time of adsorbed plasma, in the presence of 2 nM APC, 85 nM protein S, and the indicated concentrations of prothrombin. The prolongation of the clotting time represents the contribution of protein S minus that seen with APC alone.

Figure 6. Effect of increasing concentration of protein S on the inhibitory effect of prothrombin. Studies were performed using a Factor X_a recalcification of adsorbed plasma in the presence of ($\circ$) $0.4 \mu\text{M}$ or ($\bullet$) $1.6 \mu\text{M}$ prothrombin and the indicated concentrations of protein S in the presence of 1 nM APC. The prolongation of the clotting time represents the contribution of protein S minus that seen with APC alone.

$2.0 \mu\text{M}$ had no effect on the inactivation of Factor V_a by APC alone in the presence of phospholipids or platelets.

To confirm that the inhibition of protein S anticoagulant activity in plasma was solely due to prothrombin and not other molecule(s) present in plasma, prothrombin-deficient plasma was prepared as described in Methods. Laurell rocket immunoassay confirmed that the prothrombin-deficient plasma contained $< 5\%$ prothrombin compared with the starting plasma. Fig. 8 contrasts the functional activity of protein S in normal, $\text{Al}(\text{OH})_3$ -adsorbed, and prothrombin-deficient plasma. As seen, the removal of prothrombin was associated with the full expression of the cofactor potential of protein S, with the deficient plasma behaving similarly to $\text{Al}(\text{OH})_3$ -adsorbed plasma. In control studies, APC in the absence of protein S exhibited comparable anticoagulant activity in all three plasmas.

Discussion

The regulation of the anticoagulant activity of protein S has been the subject of a number of studies. Dahlback has demonstrated that 60% of the protein S in plasma was complexed to the complement binding protein C4b-binding protein (12). He and others (22) have shown that in the complex, protein S cofactor activity was lost. The physiological importance of the protein S-C4b-binding protein interaction in the modulation

Figure 7. Effect of purified prothrombin on the cofactor activity of protein S in a factor V_a inactivation assay. The reaction mixture contained 20% platelin, 2 U/ml Factor V_a , and: ($\circ$) 0.3 nM APC, (Δ) 0.3 nM APC + $0.3 \mu\text{M}$ protein S, ($\bullet$) 0.3 nM APC + $0.3 \mu\text{M}$ protein S

+ $0.5 \mu\text{M}$ prothrombin, and ($\blacksquare$) 0.3 nM APC + $0.3 \mu\text{M}$ protein S + $0.25 \mu\text{M}$ prothrombin. The volume of the reaction was adjusted to $25 \mu\text{l}$ using 20 mM Tris buffer ($\text{pH } 7.4$) containing 0.15 M NaCl, 5 mM CaCl_2 , and 0.5% gelatin. Subsamples ($2.5 \mu\text{l}$) were removed at the indicated time points and assayed for residual Factor V_a using the one-stage purified component assay described in Methods.

Figure 8. Cofactor activity of protein S in prothrombin-deficient plasma. Prothrombin-deficient plasma was prepared by immunoadsorption as described in Methods. The coagulation assay used was a Factor X_a recalcification time performed in the presence of 1 nM APC and the indicated

concentrations of protein S. The prolongation of the clotting time represents the contribution of protein S minus that seen with APC alone. ▲, normal plasma; △, prothrombin-deficient plasma; and ●, Al(OH)₃-adsorbed plasma.

of protein S activity in man remains unresolved, as the interaction between the two proteins is very slow, requiring prolonged incubation (12). It is not known whether other plasma or cellular proteins could accelerate the rate association of protein S and C4b-binding protein. Another mechanism that has been suggested for the regulation of protein S activity is the cleavage of the protein by thrombin (8–10). This reaction is unlikely to occur in vivo, because it is inhibited by physiological concentrations of Ca²⁺ and the endothelial cell protein thrombomodulin (11). Recently, we have reported that protein S binds to platelets and is cleaved and inactivated by a Ca²⁺-dependent serine protease located on the plasma membrane (13). This cellular reaction could represent another control for the activity of protein S.

The studies reported in this manuscript were initiated by the observation that the cofactor activity of protein S was more readily observed using Al(OH)₃-adsorbed plasma than with normal plasma. Mixing of normal plasma and the adsorbed plasma suggested the presence of an inhibitor directed against the functional activity of protein S. The inhibition was not against APC, since the anticoagulant activity of this protein was not affected by the type of plasma used in the assay.

The purification scheme used was aimed at uncovering the nature of the inhibitor. Several lines of evidence suggested that the inhibition was mediated by prothrombin. The inhibitor was removed after Al(OH)₃ or BaCl₂ adsorption and had identical chromatographic behavior to prothrombin. It appeared in the flowthrough of a heparin agarose column, but bound to the anion exchange resin. The elution profile from the column was very similar to that of prothrombin. The inhibitor bound to the blue Sepharose affinity column and exhibited comparable elution profile on the application of a NaCl gradient to prothrombin. Only one peak was resolved from this step that also contained inhibitory activity. Gel filtration studies confirmed that the inhibitory activity corresponded to the major protein peak. SDS-PAGE of the purified material revealed one band with a molecular mass of 72 kD identical to that of prothrombin. Prothrombin purified by conventional means and added to adsorbed plasma reproduced the inhibition of protein S activity seen with normal plasma. Further confirmation that prothrombin inhibited the activity of protein S was derived from experiments using prothrombin-deficient plasma. In these studies, the activity of protein S was significantly increased after immunodepletion of plasma from

prothrombin and was comparable to that observed in Al(OH)₃-adsorbed plasma.

The inhibitory effects of prothrombin were also observed in studies of the rates of inactivation of purified Factor V_a by APC and protein S. Unfortunately we were not able to perform detailed kinetic analysis of inhibition since human protein S exhibits limited APC cofactor activity in this system (reference 12, and our own observations). At most, a 1.5–2-fold enhancement in the rate of APC-mediated inactivation of Factor V_a could be obtained in the presence of saturating amounts of protein S. This is in contrast to the 14-fold enhancement in the cofactor activity of APC observed in assays using the Factor X_a recalcification time of Al(OH)₃-adsorbed plasma (11).

It is unlikely that prothrombin is exerting its effects merely by enhancing the rate of thrombin generation. The concentration of prothrombin used in the Factor X_a recalcification time is saturating, with higher concentrations having no appreciable effect on the clotting time or the rate of thrombin generation. Furthermore, the effects of prothrombin were not observed with APC alone, and could be reproduced in a purified component system that did not rely on thrombin generation.

In preliminary studies, we have observed that the inhibitory effects of prothrombin were much less pronounced when endothelial cells are used as a surface for the reaction compared with studies using platelets or phospholipids, further experiments are required to confirm these findings and to localize the sites on prothrombin involved in the inhibition of protein S activity.

In conclusion, the studies reported in this manuscript demonstrate that prothrombin in concentrations present in plasma, is a rapid and efficient inhibitor to the cofactor activity of protein S. Our findings suggest that these effects may represent an important regulatory pathway in the natural anticoagulant system.

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